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PRINCIPAL INVESTIGATOR: Clark W. Distelhorst, M.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 44106

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A model system for investigating the fundamental mechanism of programmed cell death, apoptosis, in breast cancer cells has been developed. This model is based on induction of apoptosis by the selective endoplasmic reticulum calcium pump inhibitor, thapsigargin. Thapsigargin induced apoptosis is inhibited by Bcl-2 and is mediated by activation of cysteine proteases belonging to the interleukin 1-ß converting enzyme family. The decision of a cell to undergo apoptosis following thasigargin treatment is regulated, in part, by the capacity of the cell to induce transcription of the grp78 stress response gene. Understanding the mechanism of apoptosis through use of this model system should provide a basis for developing new therapies for breast cancer.

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Clark Dietrich 10/15/96

PI - Signature Date

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(5) INTRODUCTION

The overall purpose of the proposal is to investigate the role of calcium in the signal transduction cascade that mediates programmed cell death, or apoptosis, in breast cancer cells. The emphasis of this proposal is on basic mechanisms. It is anticipated that an improved understanding of the mechanism of apoptosis and how it is controlled in breast epithelium should facilitate the development of novel therapeutic strategies based on inducing apoptosis.

Apoptosis is a naturally-occuring form of cell death important for the proper development and homeostasis of many tissues. Apoptosis can be induced in breast cancer cells by estrogen ablation or treatment with the antiestrogen tamoxifen, the antiprogestin RU486, or the somatostatin analogue, SMS 201-995. Understanding the mechanism of apoptosis and how it is regulated will provide an opportunity for new therapeutic initiatives, as well as insight into the pathogenesis of breast cancer. In the latter regard, there is growing evidence that aberrant cell survival resulting from inhibition of apoptosis can lead to cancer. Apoptosis is physiologically regulated by genes, such as Bcl-2. Bcl-2 is expressed in many tissues that are characterized by apoptotic cell turnover, including breast epithelium. It seems highly likely that aberrant expression of Bcl-2 may interfere with apoptosis in breast cancer cells, a concept that is being tested in our studies.

One hypothesis which this proposal addresses is that mobilization of calcium from the endoplasmic reticulum (ER) may be a critical step in the apoptotic pathway of breast cancer cells, induced by a variety of events, including hormone withdrawal, antiestrogen treatment, and growth factor withdrawal. We also predict that thapsigargin (TG), a potent and selective ER calcium pump inhibitor will induce apoptosis in breast cancer cells, bypassing more proximal steps in the apoptotic pathway, such as hormone-receptor interaction. If this prediction is correct, then it might be possible to develop new therapeutic agents for use in treating hormone-resistant breast cancer, based on the model of TG-induced cell death. Moreover, we suspect that apoptosis in breast cancer cells will be regulated by Bcl-2, or newly recognized members of the Bcl-2 family, a concept that will be tested in the current proposal.

(6) BODY OF PROGRESS REPORT

This progress report is organized according to the Statement of Work submitted with the original application.

This report contains unpublished data.

Task 1, Develop model systems for studying PCD in breast cancer, Months 1-8:

a. MCF-7 and T47D breast cancer cell lines will be acquired and optimal culture conditions will be established.

We systematically surveyed the current literature regarding available breast cancer cell lines, and also sought the advice of Dr. Gloria Heppner, Michigan Cancer Foundation. Our goal was to obtain cell lines which were reasonably well characterized and which were likely to serve as optimal models of apoptosis. Two cell lines were identified: MCF-7 and MB-MDA-468. These lines are well characterized and represent two ends of a spectrum: MCF-7 is a hormonally regulated line, which is known to express the antiapoptotic protein, Bcl-2; MB-MDA-468 is hormone independent and does not express detectable levels of Bcl-2. Although in our original Statment of Work, we intended to also use the T47D line, after a detailed survey of the literature, we concluded that the MDA-MB-468 line would fulfill our aims better. Although we have obtained the T47D line, we will at least initially hold it in reserve so that we may focus on the better characterized model systems provided by MCF-7 and MB-MDA-468.

We obtained the MCF-7 line from Dr. Stanton Gerson's laboratory at CWRU, and the MB-MDA-468 line from Dr. Marc Lippman at Georgetown University. Specific culture conditions for maintaining optimal cell viability and growth were established, according to standard published methods. Cell viability was routinely monitored by measuring trypan blue dye exclusion. The amount of apoptosis under standard culture conditions was determined by fluorescence microscopy of ethidium bromide/acridine orange stained cells. On a routine basis, we observed in each cell line that dead cells, which generally have a clearcut apoptotic morphology, detach from the plastic tissue culture dish and float in the supernatant medium, whereas adherent cells are viable and do not show morphological signs of apoptosis. Under optimal culture conditions, the number of dead cells floating in the supernatant medium was less than 10% of the total cell population (attached cells plus detached cells). In the case of MCF-7 cells, we found it necessary to supplement the tissue culture medium with insulin; otherwise, viability and growth were not optimal.

In order to develop these cell lines as model systems for studying programmed cell death, it was necessary for us to characterize the cell lines in terms of their level of expression of the antiapoptotic protein, Bcl-2, and for other members of the Bcl-2 family. Therefore, a Western blotting technique was set up to assess the level of Bcl-2 protein in MCF-7 and MB-MDA-468 cells. At this point in time, we

This report contains unpublished data.

have generated substantial data regarding Bcl-2 expression, but have not yet completed experiments charaterizing the levels of BAX and Bcl-Xl.

Under conditions of exponential cell growth, Bcl-2 was not detected in MB-MDA-468 cells but was detected by Western blotting in MCF-7 cells (Figure 1A). To develope a panel of useful cell lines for investigating the role of Bcl-2 in breast cancer programmed cell death, we stably transfected MDA-MB-468 cells with a mammalian expression vector, pSFFV-Bcl-2, encoding human Bcl-2 cDNA, or control vector, pSFFV-neo, which does not encode Bcl-2. By Western blotting, we confirmed that the Bcl-2 transfectants do indeed express substantial levels of Bcl-2 protein, while control transfectants do not (Figure 1C). These cell lines are employed in experiments described subsequently in this progress report.

Because the MCF-7 line is a hormonally responsive line, it was important for us to characterize how the level of Bcl-2 was affected by the hormonal mileau, as this could influence our studies of apoptosis in this experimental system. Therfore, we assessed the effect of estrogen withdrawal and supplementation on cell growth and the level of Bcl-2 in MCF-7 cells. To withdraw estrogen, cells were cultured in phenol red-free medium supplemented with charcoal stripped serum. (The charcoal stripping removes endogenous steroid hormones.) As shown in Figure 2, panel A, culturing MCF-7 cells in medium supplemented with charcoal extracted serum (Ext Med) significantly repressed cell growth. We assessed Bcl-2 every 48 hr after placing cells in estrogen free medium. As shown in Figure 2, panels B & C, the level of Bcl-2 declined over a period of 4-8 days following estrogen withdrawal. This was an important observation, as it suggested that the susceptibility of MCF-7 cells to programmed cell death might be influenced by the level of Bcl-2, which in turn was regulated by estrogen. The data in Figure 2 suggest that the decline in Bcl-2 expression occuring when cells were cultured in medium supplemented with charcoal stripped serum was due to estrogen withdrawal. An alternative hypothesis was that the decline in Bcl-2 was due to depletion of some other essential factor, other than estrogen, from the serum during charcoal extraction. To confirm that the decline in Bcl-2 was indeed due to estrogen withdrawal, we carried out the "add-back" experiments. In these experiments, MCF-7 cells were cultured in either regular medium or medium supplemented with charcoal extracted serum, in either the presence or absence of 1 nM 17ß-estradiol. After 6 days in culture, the Bcl-2 level was measured by Western blotting and expressed as fold induction (level of Bcl-2 + estradiol / level of Bcl-2 - estradiol) (Figure 3). The data indicate that estradiol indeed induces Bcl-2 expression, and that the level of Bcl-2 in cells cultured in medium supplemented with extracted serum was restored by supplementing the

This report contains unpublished data.

serum with 1 nM 17ß-estradiol. Hence, we conclude that the decline in Bcl-2 expression observed when cells are cultured in medium supplemented with charcoal extracted serum is, at least in large part, due to estrogen withdrawal. Moreover, consistent with the growth stimulatory effect of estrogen on MCF-7 cells, supplementation of charcoal extracted serum with 1 nM 17ß-estradiol restored cell proliferation (Figure 4).

The data in Figure 2 also indicate that the level of Bcl-2 increased approximately two-fold when cells were cultured for 8 days in the presence of unextracted serum. Thus, the data in Figure 2 suggest that there is a correlation between cell proliferation and Bcl-2 expression. To test this correlation, independent of the influence of hormonal manipulations, we tested the relationship between cell proliferation and Bcl-2 expression in the hormone-independent MDA-MB-468 cell line. In Figure 1, panel B, the level of Bcl-2 in MDA-MB-468 cells was measured by Western blotting over a period of 4 days after cells reached confluency. Within 1 day of reaching confluency, Bcl-2 was readily detectable in the cells, whereas Bcl-2 was not detected during exponential growth. These data, coupled to those described in Figure 2, indicate that Bcl-2 expression correlates directly with cell density, suggesting that Bcl-2 expression may be regulated by cell-cell contact or by autocrine factors which increase with increased cell density.

In summary, Task 1a was completed, establishing two well characterized human breast cancer cell lines for use in our work. One cell line, MCF-7 was found to express Bcl-2 in an estrogen-regulated fashion. The other cell line, MB-MDA-468, was found to express Bcl-2 when allowed to remain at confluence, but not during exponential growth.

b. Methods of inducing PCD will be established, using both hormonal and non-hormonal manipulations.

To determine whether or not estrogen withdrawal induces apoptosis in MCF-7 cells, we cultured the cells in the presence of charcoal-extracted serum for up to 8 days. This corresponds to the time period over which we characterized Bcl-2 expression and cell growth under Task 1a. Cells were stained with ethidium bromide and acridine organge and then examined for apoptotic morphology by fluorescence microscopy. No morphological evidence of apoptosis was observed. Furthermore, we analyzed cellular DNA derived from cells at various time points, up to 8 days during culture in charcoal-extracted serum. There was no evidence of DNA fragmentation, consistent with the conclusion that the cells did not undergo apoptosis upon estrogen

withdrawal. Hence, although cell growth was hormonally regulated, there was no evidence that the cells underwent apoptosis upon hormone withdrawal, even though the level of Bcl-2 expression declined during this period of time.

We conclude that the level of Bcl-2 present in cells after hormone withdrawal is probably sufficient to inhibit apoptosis. This hypothesis will need further testing, as alternative hypotheses exist that would explain our findings. Nevertheless, we have concluded that spontaneous apoptosis does not occur in MCF-7 cells following estrogen withdrawal. Therefore, we sought a better way of inducing apoptosis, which led directly into Task 3, where we have found that thapsigargin treatment is an effective way of inducing apoptosis in breast cancer cells (see below). Please note at this point we decided to begin work on task 3 before task 2 was completed, so as to test the strength of our model system and to make more efficient progress. Thus, task 3 was given higher priority than task 2, although task 2 was initiated during this first year of funding.

c. PCD assays, including DNA fragmentation assays, will be applied to breast cancer cells undergoing PCD.

Our laboratory is proficient at two apoptosis assays: DNA fragmentation by agarose gel electrophoresis and morphological assessment by fluorescence microsopy of ethidium bromide and acridine organge stained cells. We have successfully applied both of these techniques without difficulty to the breast cancer cell systems described in this progress report. These assays are sufficient for the studies currently underway in Tasks 1-3 of this proposal. These assays are efficient, reliable and inexpensive. Thus, they will be utilized in our work, unless need for other types of assays develops.

Task 2, Adapt established calcium assays to breast cancer cell system, Months 4-12:

For reasons discussed above, we elected to move directly into Task 3, before completing Task 2. This was possible, because Task 3 is not dependent upon the findings in Task 3. In fact, in retrospect, it would have been more logical to place Task 3 ahead of Task 2 in our original application. Under Task 2a, we have begun to work out methods for loading breast cancer cells with Fura-2 AM, and to assess intracellular calcium by fluorometry, but data is insufficient to present at the present time.

This report contains unpublished data.

Task 3, Determine effect of thapsigargin on growth and viability of breast cancer cells, Months 12-18:

a. Measure effects of thapsigargin treatment on growth and viability of breast cancer cells over a broad dose-response range.

We wished to determine the effect of the endoplasmic reticulum (ER) calcium pump inhibitor, thapsigargin (TG), on cell growth and viability in breast cancer cells. Therefore, MCF-7 and MDA-MB-468 cells were treated with a range of concentrations of TG, known from previous studies to inhibit calcium uptake by the ER. We found that 100 nM was sufficient to inhibit cell proliferation. In both cell lines, treatment with 100 nM TG slowed cell growth and induced loss of adherence properties. In MDA-MB-468 cells, treatment with 100 nM TG caused cell death, measured by increased incorporation of trypan blue dye, whereas TG treatment did not appear to induce cell death in MCF-7 cells. Based on these initial observations, we proceeded directly to the next planned experiments, designed to measure morphological and biochemical parameters to determine whether thapsigargin-induced loss of viability is due to induction of apoptotic cell death.

b. Measure morphological and biochemical parameters to determine whether thapsigargin-induced loss of viability is due to induction of apoptotic cell death.

Both MCF-7 and MDA-MB-468 cells were treated with TG, an ER calcium pump inhibitor that has been shown previously to induce apoptosis in lymphoma cells and prostate cancer cells (1,2). Nuclear chromatin condensation, a hallmark of apoptosis, was readily detected by fluorescence microscopy in TG-treated MDA-MB-468 cells, but was not detected in TG-treated MCF-7 cells (Figure 5, panel A). In this figure, cells were treated with 100 nM TG for 48 h. In contrast to MDA-MB-468 cells, MCF-7 cells were resistant to TG-induced apoptosis, whether or not they were cultured in charcoal extracted serum to remove endogenous estrogen (Figure 5, panel A). Thus, culturing MCF-7 cells under estrogen-free conditions did not increase their susceptibility to TG-induced apoptosis, indicating that the decrease in Bcl-2 level associated with estrogen deprivation is insufficient to increase the susceptibility of MCF-7 cells to apoptosis induction by TG. To assess the effect of exogenous Bcl-2 in MDA-MB-468 cells, 468-neo and 468-Bcl-2 cells (see Figure 1, panel C) were treated for 48 h with 100 nM TG. TG-induced apoptosis was observed within 48 h following addition of 100 nM TG to 468-neo cells, whereas apoptosis was not observed in 468-Bcl-2 cells (Figure 4, panel B).

Recent findings indicate that apoptosis in general is mediated by proteases related to interleukin 1-ß converting enzyme (ICE). ICE, along with as many as nine

This report contains unpublished data.

ICE-like proteases, compose a family of cysteine proteases that characteristically cleave proteins at aspartic acid residues (3,4). These proteases fall into three subfamilies: the Ced-3 family, which includes the mammalian protease CPP32 (also known as Yama or apopain); the ICE family; and the NEDD-2 family. Recent findings suggest that at least some of these proteases operate within an amplifiable protease cascade, culminating in activation of CPP32 (5,3). Cleavage of selected target proteins by CPP32 and related proteases appears to be directly responsible for the stereotypic morphological changes characteristic of apoptosis (5). We therefore employed two strategies to determine whether or not TG-induced apoptosis was mediated by ICE-like proteases. In the first strategy, MDA-MB-468 cells were stably transfected with a cDNA encoding p35, a baculovirus inhibitor of ICE-like proteases. Although p35 protein was not detected in lysates of stably transfected cells by Western blotting (not shown), p35 mRNA was readily detected by RT-PCR. Control, vector only, transfectants and p35 transfectants were examined before and after TG treatment to detect morphological changes typical of apoptosis (Figure 6, panel A). The proportion of apoptotic cells was quantitatd by examining ethidium bromide/acridine orange stained cells under fluorescence microsopy. The results indicate that p35 inhibits TG-induced apoptosis.

In a second approach at testing the importance of ICE-like proteases in TG-induced apoptosis, cells were treated with TG in the presence or absence of the peptide fluoromethylketone inhibitor Z-VAD-fmk. The tripeptide sequence in Z-VAD-fmk corresponds to the P1 to P3 residues of the pro-IL-1ß cleavage site (Tyr4ValAlaAsp1Gly), where ICE cleaves between the Asp and Gly residue (6). Deletion of the Tyr residue would be expected to broaden the inhibitory spectrum to include not only ICE, but other proteases closely related to ICE. An effective treatment regimen was empirically derived in which cells were treated with 200 μM doses of Z-VAD-fmk added 1 h prior to TG, and every 12 h thereafter over a period of 48 h. In addition to Z-VAD-fmk, Z-FA-fmk, a cathepsin inhibitor, was employed as a negative control. Apoptosis induction by TG, measured by fluorescence microscopy, was inhibited by Z-VAD-fmk, but not the control inhibitor, Z-FA-fmk (Figure 6, panel B), thus confirming that ICE-like protease activity is necessary for mediating apoptosis in response to TG treatment.

c. Determine if cells adapt to growth in thapsigargin, and if cells so adapted become resistant to induction of PCD by hormonal manipulations.

This task is not as high a priority as other tasks, and therefore its initiation has been delayed.

This report contains unpublished data.

d. Measure other parameters of thapsigargin-induced endoplasmic calcium pool depletion, including transcriptional induction of grp78 and grp94 genes, alterations of protein processing, and capacitative calcium entry.

Although the precending findings clearly indicate that TG induces apoptosis in breast cancer cells, we observed that the onset of cell death is much slower than in lymphoid cells. For example, we have found that certain lymphoma cell lines undergo apoptosis within less than 12 h following addition of 100 nM TG (1). In contrast, only a modest increase in apoptotic cells is observed within the first 24 after adding 100 nM TG to MDA-MB-468 cells. To examine the mechanism underlying differences in the susceptibility of different cell types to apoptosis induction, we have investigated the grp78/grp94 stress response to TG-induced ER calcium pool depletion in various cell types.

The ER is the major intracellular reservoir of calcium in non-muscle cells. The ER calcium pool is essential for a number of vital cellular functions which include protein processing within the ER, maintaining high translation rates of newly transcribed messages, preserving the structural integrity of the ER, and regulating cell proliferation and cell cycle progression. Under physiological conditions, the ER calcium pool is maintained by an associated calcium-ATPase that pumps calcium into the ER lumen from the cytoplasm. The ER calcium pool can be depleted by treating cells with the calcium ionophore A23187 or the selective ER calcium-ATPase inhibitor thapsigargin (TG).

ER function is mediated, in part, by intraluminal calcium binding proteins which include the glucose regulated proteins (GRP's) GRP78 and GRP94. GRP78 and GRP94 are found constitutively within the ER, and transcription of the genes for these proteins is elevated in response to malfolded proteins, inhibition of glycosylation and ER calcium pool depletion. GRP78 is a highly conserved 78 kDa protein that shares 60% amino acid homology with the 70 kDa heat shock protein (HSP 70). GRP78 (also known as BiP) associates transiently with nascent proteins as they traverse the ER and aids in their folding and transport. The binding of immature proteins by GRP78 requires ATP, and GRP78 has both ATP binding and ATPase activities. GRP94 is a 94 kDa glycoprotein that shares 50% amino acid homology with HSP90. GRP94 acts in concert with GRP78 to fold nascent proteins, and also exhibits ATPase activity.

In epithelial cells and fibroblasts, grp78 and grp94 are coordinately regulated through common calcium-responsive promoter elements that respond to ER calcium pool depletion. Thus, ER calcium pool depletion, induced by either A23187 or TG, signals an increase in grp78/grp94 transcription, producing a 5- to 20 fold

This report contains unpublished data.

elevation of grp78/grp94 mRNA levels. In these cells, the loss of ER calicum induced by TG or A23187 does not result in a loss of viability, unless the grp78/grp94 stress response is repressed by antisense, promoter competition or ribozyme techniques. Moreover, grp78/grp94 induction restores protein synthesis under conditions where intracellular calcium is depleted. This indicates that grp78/grp94 gene induction is a protective response mechanism by which cells accommodate to potentially lethal stress caused by the disruption of intracellular calcium homeostasis.

Based on this background information, we reasoned that the decreased susceptibility of breast cancer cells to apoptosis induction by TG, compared to lymphoma cells, might be secondary to differences in grp78/grp94 stress response induction. To test this hypothesis, we measured the level of grp78 mRNA following TG treatment in WEHI7.2 lymhoma cells and several breast cancer cell lines, Mm5MT, MCF-7, and MDA-MB-468. We found that TG induced calcium loss from the ER of WEHI7.2 cells does not induce grp78 transcription, even if cells are protected from undergoing apoptosis by overexpressing Bcl-2 (W.Hb12 is a subclone of WEHI7.2 cells stably transfected with cDNA encoding Bcl-2) (Figure 7). In contrast, grp78 transcription was strongly induced in each of the breast cancer cell lines (Figure 8). In summary, these findings have two important implications. First, the grp78 stress response may be differentially regulated among different types of cells, with a much greater response observed in non-lymphoid cells than in lymphoid cells. Second, regulation of the grp78 stress response may be a major factor in deciding whether a cell lives or dies in response to disruption of intracellular calcium homeostasis. Indeed, the absence of a calcium-mediated grp78 stress response may be the basis for the marked susceptibility of certain lymphoma cells to TG-induced apoptosis, and the decreased susceptibility of breast cancer cells to TG-induced apoptosis.

(7) CONCLUSIONS

Task 1. The MCF-7 and MB-MDA-468 human breast cancer cell lines will provide an excellent model system for carrying out the proposed research.

a. MCF-7 represents a hormone-responsive line that expresses significant levels of Bcl-2.

b. In MCF-7 cells, the level of Bcl-2 can be down-regulated by estrogen withdrawal, and induced by estrogen supplementation.

c. In MCF-7 cells, the downregulation of Bcl-2 following estrogen withdrawal is not associated with apoptosis.

d. MB-MDA-468 cells do not express Bcl-2, unless they have been maintained at confluence for several days.

This report contains unpublished data.

e. In breast cancer cells (both MCF-7 and MB-MDA-468), the level of Bcl-2 expression correlates with the phase of cell growth.

f. Morphological assessment, by fluorescence microscopy of acridine orange and ethidium bromide stained cells, is a feasible and efficient method for detecting apoptosis in the breast cancer cell lines we are using.

Task 3. The intracellular calcium pump inhibitor, thapsigargin, induces apoptosis in breast cancer cells, and thapsigargin-induced apoptosis will be an excellent model system for studying the role of calcium in mediating programmed cell death in breast cancer.

a. Thapsigargin (TG) induces growth arrest of MCF-7 cells, without significant loss of viability, due to the significant level of Bcl-2 in MCF-7 cells.

b. Estrogen withdrawal does not increase the susceptibility of MCF-7 cells to TG-induced apoptosis, suggesting that the reduction in the level of Bcl-2 following estrogen withdrawal is insufficient to render cells susceptible to apoptosis.

c. TG-induces marked loss of viability in MDA-MB-468 cells.

d. TG-induced cell death in MDA-MB-468 cells is accompanied by typical morphological features of apoptosis.

e. TG-induced apoptosis of MDA-MB-468 cells is mediated through activation of ICE-like proteases and is inhibited by overexpression of the baculovirus p35 protein or by treatment with Z-VAD-fmk, both of which inhibit ICE-like protease activities.

f. Although TG induces apoptosis in MDA-MB-468 breast cancer cells, the induction of cell death is much slower than in lymphoma cells.

g. MDA-MB-468 and Mm5MT breast cancer cells induce transcription of the stress response gene, grp78, in response to TG-induced ER calcium release, whereas the lymphoma cell do not. Thus, induction of grp78 stress response may be a factor that regulates whether or not a cell undergoes apoptosis in response to TG treatment. The decreased susceptibility of breast cancer cells to apoptosis induction following TG treatment, compared to lymphoma cells, may be due to the inherent capacity of breast cancer cells to mount a grp78 stress response.

Implications of the research

During the first two years of support, this laboratory has made significant progress toward the long term goal of the research, which is to understand the programmed cell death process in breast cancer cells so that new therapeutic strategies can be developed. In partial fullfillment of Technical Objective C2 of the original proposal, we have discovered that the intracellular calcium pump inhibitor, thapsigargin, induces apoptosis in a human breast cancer cell line. This

This report contains unpublished data.

finding is significant because it indicates that calcium mediated signal transduction pathways that mediate apoptosis are active in human breast cancer cells, indicating that it may be feasible to develop novel forms of therapy based on triggering programmed cell death through a calcium-mediated signal. In partial fulfillment of Technical Objective C3, we have found that TG-induced cell death is inhibited by Bcl-2. This will allow us, in future years of support, the opportunity to test hypotheses, outlined in the original proposal, regarding the mechanism of Bcl-2 action in breast cancer cells. Moreover, the observation that TG induces apoptosis, and that TG-induced apoptosis is inhibited by Bcl-2, sets the stage for pursuit of Technical Objective 4, in which we will investigate how endoplamsmic reticulum calcium release signals programmed cell death in breast cancer cells. Most importantly, we have validated our TG-induced model of apoptosis by showing that TG-induced apoptosis is mediated by ICE-like proteases. We are among the first laboratories to stably express the baculovirus p35 apoptosis inhibitory protein in breast cancer cells to prove that apoptosis is indeed mediated through ICE-like protease activation.

There will be no significant changes from the original plans of research and the specific aims of the original research proposal will be followed during the third year of support.

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(9) APPENDICES

The appendix contains 6 figures referred to in the Body of the Progress Report.

Figure 1. Bcl-2 expression in MDA-MB-468 cells.

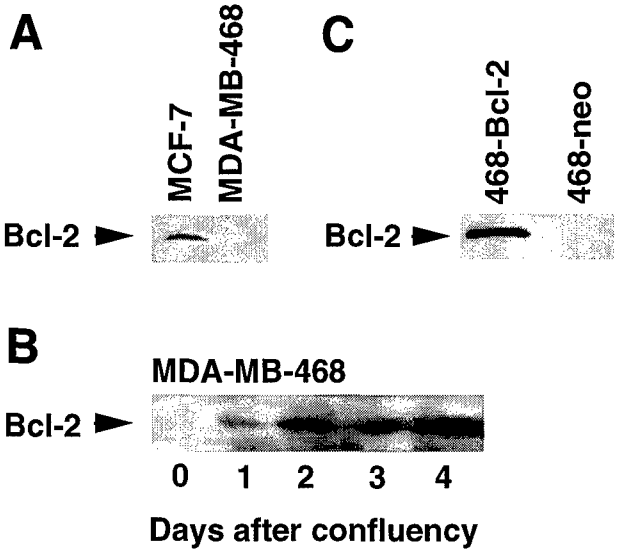


Figure 2: Effect of estrogen on Bcl-2 expression in MCF-7 cells.

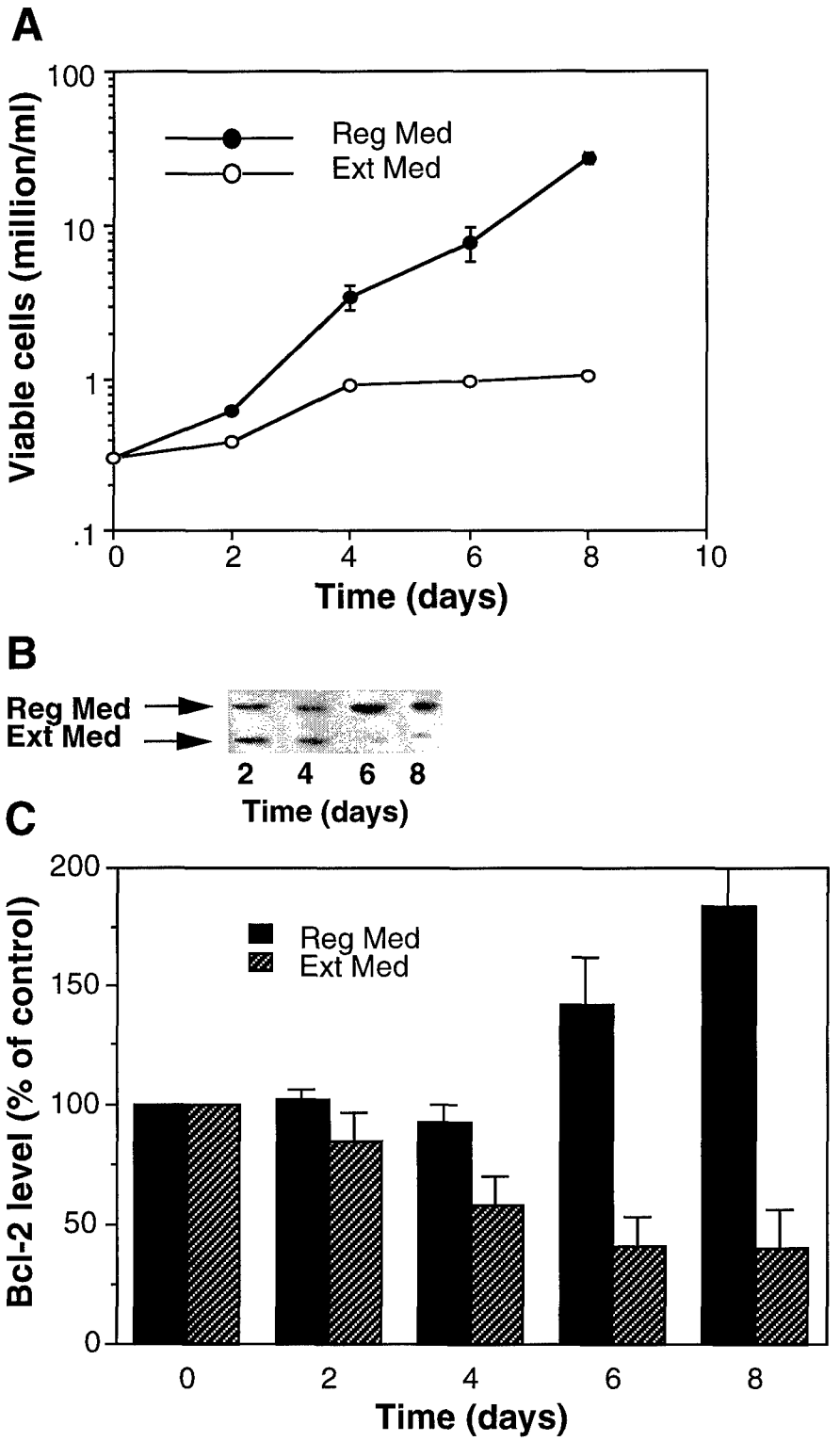


Figure 3: Effect of estrogen restoration on Bcl-2 expression in cells cultured in regular medium versus extracted medium.

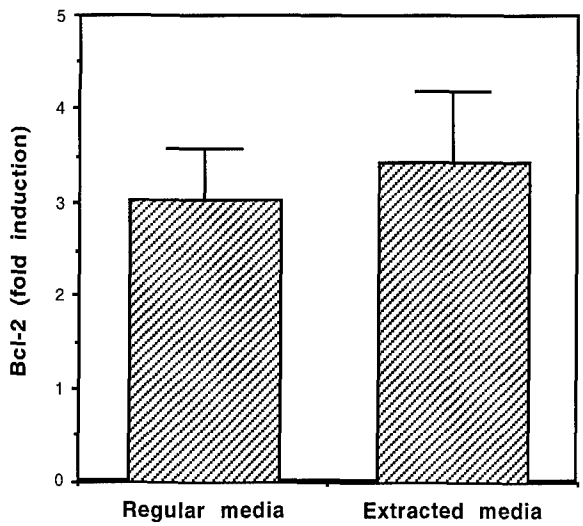


Figure 4: Effect of estrogen supplementation on cell proliferation for MCF-7 cells cultured in either regular medium (Reg) or medium supplemented with charcoal extracted serum (Ext).

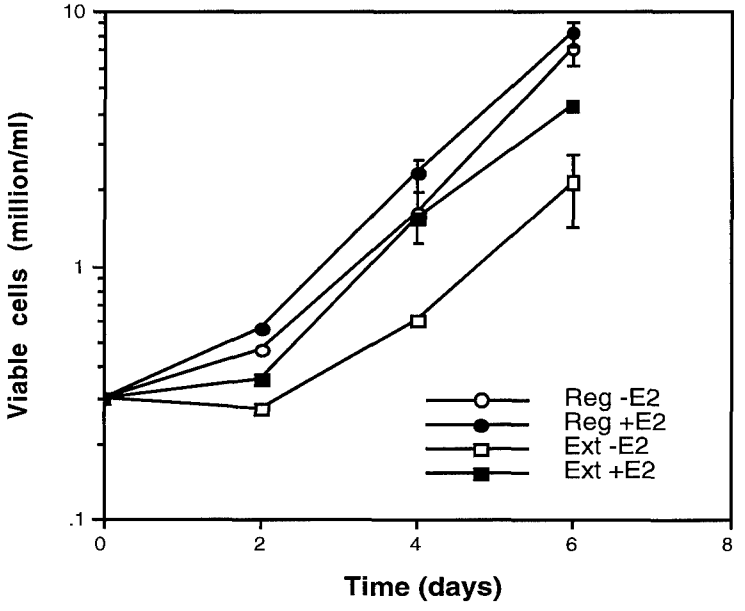


Figure 5: Inhibition of TG-induced apoptosis by Bcl-2

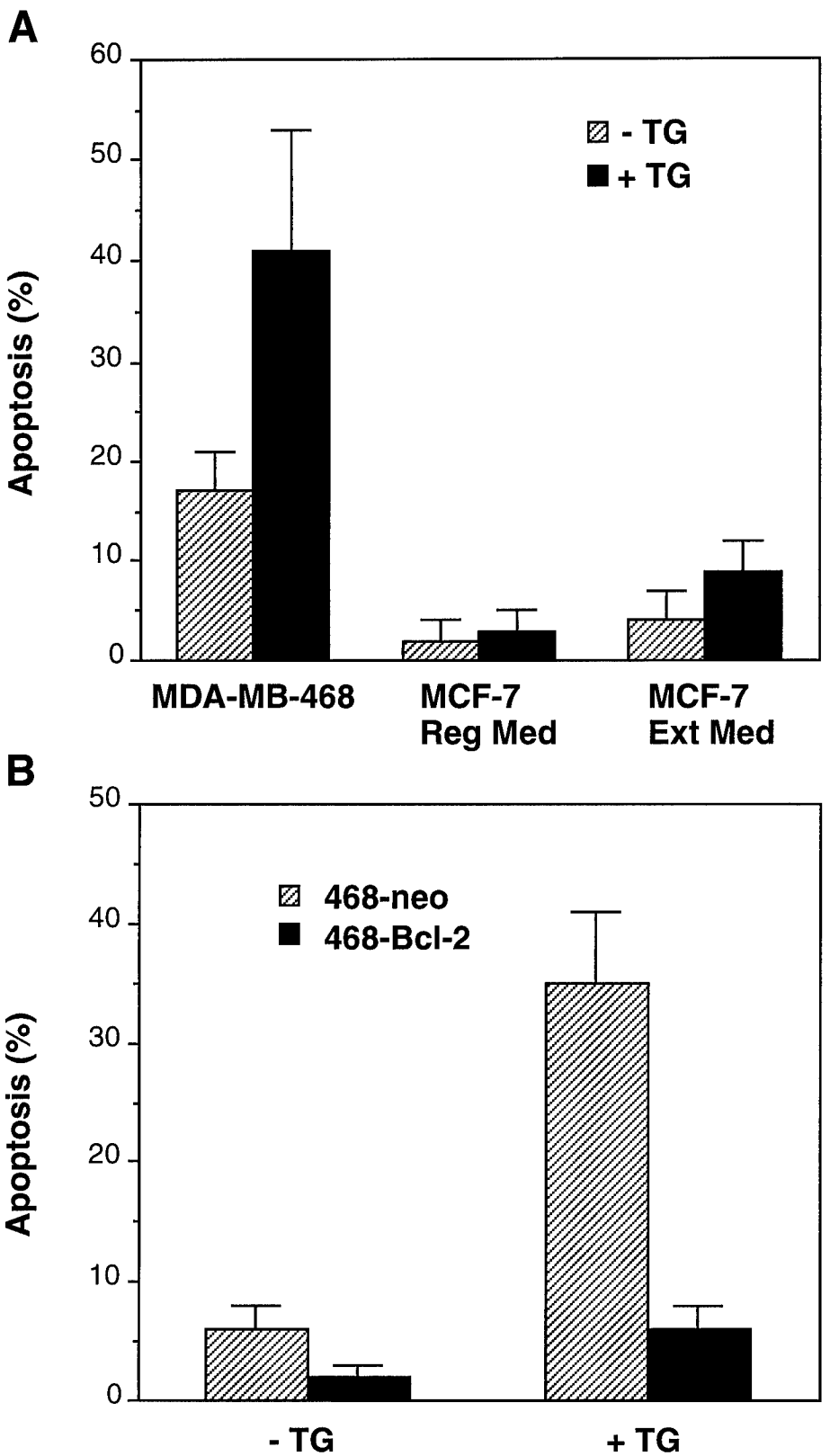
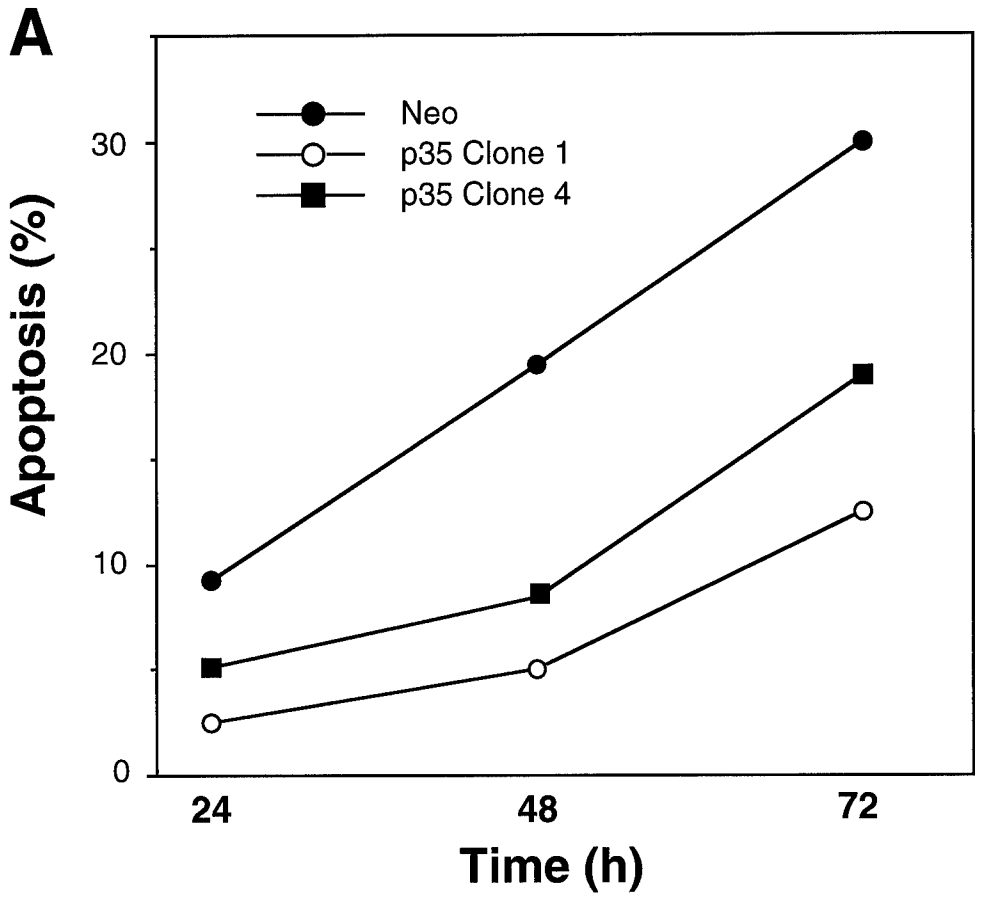


Figure 6: Effect of p35 and Z-VAD-fmk on TG-induced apoptosis



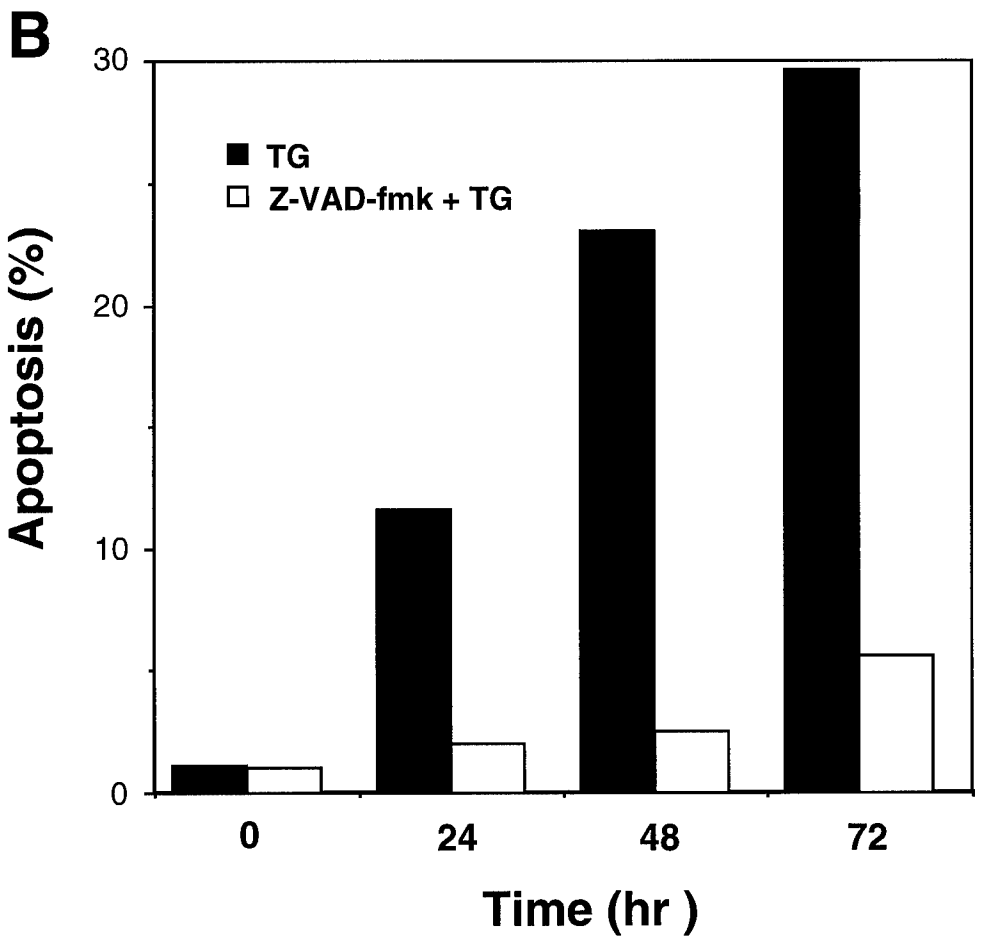


Figure 7. Effect of TG on grp78 mRNA levels by Northern hybridization.

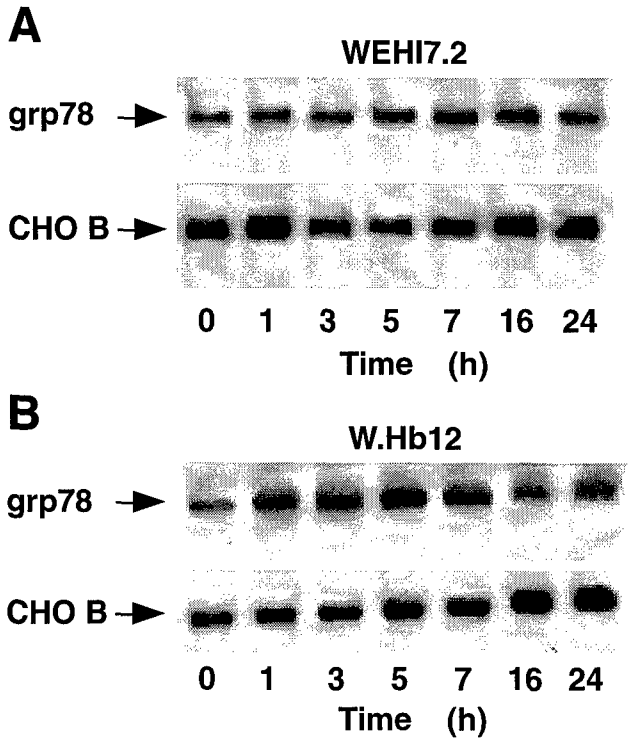
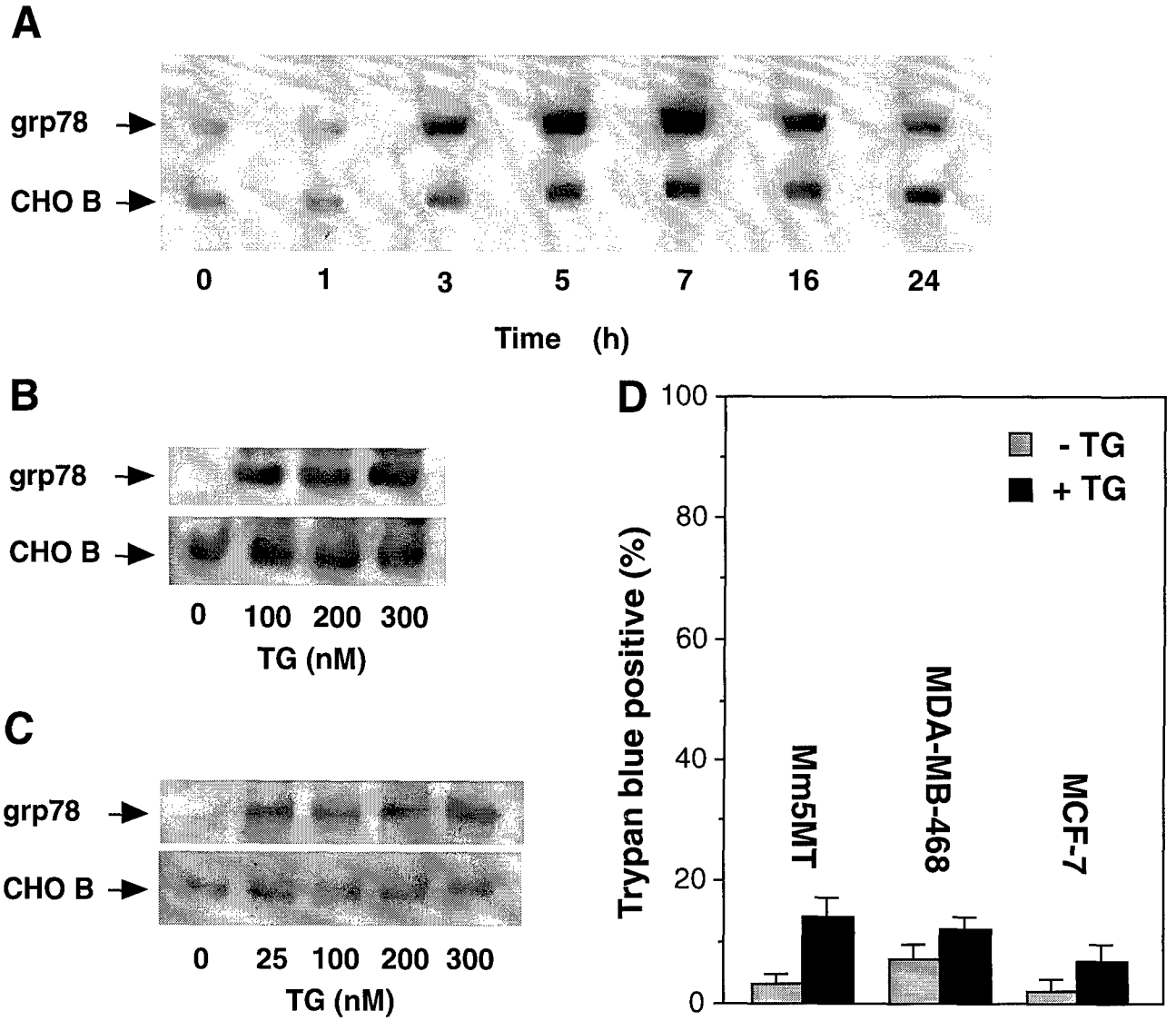


Figure 8: Effect of TG on grp78 mRNA levels by Northern hybridization.



Rec'd 1/11/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Grants. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

94-J-4451 AD-B221966

completed 1-14-00 ca